

Infection and drug resistance of *Streptococcus agalactiae* among perinatal pregnant women in Xinjiang

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Abstract

Background: Group B streptococcus (*Streptococcus agalactiae*) is one of the most common pathogens causing meningitis, bacteremia and pneumonia. The drug resistance mechanisms of group B streptococcus in different countries and regions also show regional differences.

Method: The study population was comprised of 1877 pregnant women of 34-38 weeks who underwent prenatal examination in the gynecology and obstetrics outpatient clinic of Xinjiang People's Hospital, between January 1, 2019 and January 31, 2020. Clinic specimens were collected and identified by the API bacteria Rapid Identification card for the downstream group B Streptococcus (*Streptococcus agalactiae*) isolation. Drug susceptibility of the *Streptococcus agalactiae* isolated was detected by Kirby-Bauer disk diffusion method. Macrolide–lincosamide–streptogramin B (MLS_B) resistance was determined by D test. Real-time quantitative polymerase chain reaction and Gene sequencing was performed for the resistance genes *ermA*, *ermB*, *mreA*, *erm* (47), *mefA/E* and *Lin B*.

Results: 149 *Streptococcus agalactiae*-positive strains were identified by clinical isolation, with a positive rate of 7.94%. Group B Streptococcus showed 100% susceptibility to linezolid, penicillin, vancomycin, meropenem, ampicillin, ceftriaxone, 44.97%, 35.57%, 56.38% and 29.53% susceptibility to levofloxacin, erythromycin, tetracycline and clindamycin, respectively. Among the 149 isolates, 127 strains showed macrolide resistance phenotype. The detection rate of intrinsic resistance phenotype (cMLS) was 40.94% (59/127), active efflux resistance phenotype (MS) 9.45% (12/127), and induced resistance phenotype (iMLS) 22.83% (29/127).

Conclusion: The *ermB* gene-mediated 50s ribosome target site change co-existing with *mreA* gene for macrolide resistance efflux may play a major role in the mechanism of *Streptococcus agalactiae* resistance macrolide resistance of in perinatal women in Xinjiang. The change of 50s ribosomal target site mediated by *ermB* gene may be the main reason for drug cross-resistance.

Introduction

Group B Streptococcus (GBS), also named as *Streptococcus agalactiae*, normally located in the vagina of women and the intestines of human body, and the bacterial carrier rate can reach about 30%[1, 2]. Vaginal *Streptococcus agalactiae* can go up to the fetal membrane to cause the release of proteolytic enzymes and inflammatory factors, so that the local edema and tension of the fetal membrane are reduced, resulting in premature rupture of the fetal membrane, when immunity of pregnant women is reduced[3]. Premature rupture of the fetal membrane makes it easy for various pathogens to enter the uterine cavity to intrauterine infection, and releases inflammatory factors to stimulate the contraction of uterine smooth muscle, resulting in premature delivery, abortion and neonatal dysplasia[3-6].

Generally, β -lactams are the preferred drugs for the treatment of *Streptococcus agalactiae* infection, but for allergic patients, macrolides and related drugs are effective alternatives. At present, the mainly known mechanism of macrolide antibiotics inhibiting Streptococcus is the target modification of 23s rRNA

methylase encoded by [7] ribosome methylation erm gene[8]. The methylation of rRNA can lead to the change of ribosome configuration, resulting in the decrease in the binding ability of macrolides, glycosamides, and streptogramins B to bacterial ribosome targets[7]. Since these antibiotics have the same or overlapping target sites, bacteria can simultaneously cross-resistant to these three types of antibiotics, known as macrolide–lincosamide–streptogramin B (MLS_B) resistance[8, 9]. The common drug-resistance related genes in Streptococcus are ermA, ermB, and ermC[10].

MLS_B resistance can be divided into intrinsic resistance (cMLS_B) and inducible resistance (iMLS_B)[11]. The drug resistance phenotype of iMLS_B is usually resistant to 14 and 15-membered macrolides such as erythromycin, and sensitive to lincosamides, streptomycin B, and partial sensitive to 16-membered macrolides[12]. In addition, the resistance to clindamycin can be expressed in the presence of erythromycin. This is because ermC resistance gene phenotype can be induced by a translation attenuation mechanism in erythromycin-exposed environment[13]. Strains with induced MLS_B resistance usually show low expression level of methylase mRNA, but in the presence of inducers, the mRNA transcription is activated and leads to over-expression of methylase, resulting in cross-resistance of bacteria to all 14, 15 and 16-membered macrolides and lincosamides[14].

Since the late 1980s, a new phenotype, the M-type, has been found in a group of streptococcus and pneumococci isolated in many countries. It includes resistance to 14- and 15-member macrolide antibiotics, but is sensitive to 16-membered macrolides, streptococcus[15]. This drug resistance mechanism is based on proton-dependent efflux system, encoded by mef gene: a group of streptococci mefA and pneumococcal mefE, the homology is 90%[16]. Recently, mef gene was also found in group C streptococcus. Preliminary studies have shown that they may be widespread in other Streptococcus, including Streptococcus agalactiae (Group B Streptococcus, GBS)[17]. In addition, a new efflux system encoded by mreA, unlike the Mef pump[18], has been characterized in a single Streptococcus agalactiae strain resistant to 14, 15 and 16-membered macrolides[9, 14, 17].

At present, some European and American countries have carried out prenatal Streptococcus agalactiae screening for pregnant women and antibiotic treatment during pregnancy, reducing the incidence of neonatal Streptococcus agalactiae[19]. Antibiotic prevention preferred penicillin, if pregnant women with penicillin allergy, can choose ampicillin, cefazolin, clindamycin, etc. It is reported that the drug resistance rates of Streptococcus agalactiae to clindamycin and erythromycin increased year by year[8, 18, 20].

In this study, 34 to 38 gestational week pregnant women of reproductive age were screened to explore the perinatal Streptococcus agalactiae infection in Xinjiang region. The Streptococcus agalactiae isolates were characterized by phenotypic and molecular methods including D-test, conventional PCR and gene sequencing to detect the major erythromycin and clindamycin resistance genes.

Materials And Methods

Ethical approval

The study protocol was approved by the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region.

Bacterial strains and clinical samples

A total of 1877 pregnant women (aged 18–40 years old) at 34-38 gestational weeks of prenatal examination attending the obstetrics and gynecology clinic of Xinjiang People's Hospital were selected as the research objects. The procedure for collection was explained to each patient before specimens were taken. The secretions of low vaginal segment and anal sphincter were collected and inoculated on standard MacConkey agar with 5 % sheep blood at 37°C in 5% CO₂ for 24 hours[10, 13]. For quality control, *Streptococcus agalactiae* ATCC 12403 were used as control strains. All clinical samples were collected from January 2019 until January 2020.

149 strains of *Streptococcus agalactiae* were isolated and identified by automatic microbial identification and drug sensitivity analysis system, the API bacteria Rapid Identification card (bioMerieux Inc., France). *Streptococcus agalactiae* strains isolated were numbered and recorded as G1 ~ G149, and preserved in ultra-low temperature refrigerator for subsequent experiments.

Antimicrobial susceptibility testing

The antimicrobial susceptibility to ampicillin (10µg), vancomycin (30µg), linezolid (30µg), penicillin (10µg), erythromycin(15µg), levofloxacin(5µg), meropenem (10µg), tetracycline(30µg), ceftriaxone(30µg) and clindamycin(5µg) of the group B streptococcus isolates, was determined by Kirby-Bauer disk diffusion method. The results were interpreted according to CLSI 2018 M100-28th guidelines.

Identification of MLS_B resistant phenotypes

MLS_B resistance phenotypes of the *Streptococcus agalactiae* isolates were investigated by the D-test method using erythromycin (15µg) and clindamycin (2µg) disks at 37°C in 5% CO₂ for 24 hours at a distance of 15mm. After incubation, samples that showed flattening of the clindamycin zone of inhibition adjacent to the erythromycin disk were considered to be inducible MLS_B (iMLS_B). Samples resistant to both antibiotic disks were taken as intrinsic MLS_B (cMLS_B), while as MS (macrolide streptogramin) phenotype when resistant to erythromycin and sensitive to clindamycin with a negative D test.

Genome DNA preparation

The Group B streptococcus colonies on the blood agar plate were picked, and genomic DNA was isolated using the QIA symphony DSP Virus/Pathogen kit in the QIA symphony system according to the manufacturer's instructions (Qiagen, USA). The purified genome DNA solution was stored at -20°C for later use[5].

Erythromycin and clindamycin resistance genes detection

The macrolides antibiotics resistance related genes, *mreA*, *mreB*, *mefA*, *mefE*, *ermB*, *ermA* and one gene for clindamycin resistance *linB* were investigated by the conventional PCR method. The amplification conditions for amplification were the following: initial denaturation for 2 minutes at 95°C, followed by 35 cycles consisting of denaturation at 95°C for 60 seconds, annealing at 57°C for 60 seconds, extension at 72°C for 5 minutes, and a final extension at 72°C for 10 minutes. A list of primers sequences and lengths of the amplified products used in this study are shown in **Table 1**. The amplified products were analyzed by electrophoresis on 1%(w/V) agarose gel in TAE buffer. Negative control reactions were carried out simultaneously without any template DNA. The primers and PCR conditions followed previously described methods[21].

Nucleotide sequencing and Bioinformatic Analysis

The PCR products of iMLS_B and MS group were recovered and purified by DNA Purification Kit (Beyotime, PR China). The identity of the amplicons was confirmed after determination of the nucleotide sequencing performed by Shanghai Sangon Technology Company. Comparative analysis of antibiotics resistance related genes *ermB* and *mreA* is detailed in the **Supplementary 1**.

Statistical analysis

All data were collected from at least 3 biological replicates and performed out at least technical duplicate. Statistical analyses were performed using GraphPad Prism, version 7.03. P values of <0.05 were considered statistically significant.

Results

Baseline characteristics

All statistical results have been considered elimination of the *Streptococcus agalactiae* strains isolated from the same part of the patient. Up to 1877 pregnant women of 34-38 gestational weeks were enrolled in this study. The mean age of the patients was 29±5 years. 149 strains of *Streptococcus agalactiae* were isolated from these clinical samples, genital tract secretion (100, 67.11%) and anal secretion (49, 32.89%), the colonization rate was 7.94% (149/1877).

Macrolides susceptibility

Streptococcus agalactiae isolates were tested for antimicrobial susceptibility to ten antimicrobials (linezolid, penicillin, vancomycin, meropenem, ampicillin, ceftriaxone, levofloxacin, erythromycin, tetracycline, and clindamycin) using the disk-diffusion method. Among 149 GBS isolates, 127 strains (85.23 %) showed macrolide resistance phenotype. The details of susceptibility profile of the *Streptococcus agalactiae* isolates (n=149) were listed in **Table 2**. And the macrolide-lincosamide-streptogramin B (MLS_B) phenotypes were counted in **Table 3**. The D test result of the MLS_B phenotype is showed as in **Figure 1**.

Macrolide Resistance genes and efflux pump genes analysis

The sensitivity and specificity of the D-zone test to detect inducible MLS_B is in concordance with that of erythromycin/clindamycin resistance gene detection by polymerase chain reaction (**Table 4**). 149 clinical isolates contained 93 strains of erythromycin resistant GBS, erythromycin resistance rate was 62.42%. D-test results showed that 52 strains of cMLS_B, 29 strains of iMLS_B, 12 strains of MS phenotype and 34 strains of L phenotype. Among the 93-erythromycin resistant GBS strains, 79 strains highly expressed the erythromycin resistance gene ermB, with a percent of 84.95% in total ninety erythromycin resistance strains, while ermA gene 6.45% (6/93), and mreA, 93.55% (87/93). It is worth noting that the recently reported erm (47) resistant efflux pump gene, 3.23% (3/93) was found in GBS isolated from perinatal women in Xinjiang. MLS_B resistance phenotype and erythromycin/clindamycin resistance gene correlation statistical analysis is shown in **Table 5**.

Discussion

Group B streptococcus is the main pathogen causing perinatal infection in women, and may cause urinary infection, pneumonia infection and diabetes infection in the elderly[2, 22]. At present, the infection rate of group B streptococcus among perinatal pregnant women and newborns in China is very high[23], so the prevention and detection of group B streptococcus should be carried out[24]. The drug resistance mechanism of Group B streptococcus to erythromycin mainly has two aspects[25]. The first is that Group B streptococcus can rely on energy efflux pumps. The second is that 23rRNA methylase can catalyze 50s ribosome and change the target position[13, 23]. Methylase is coded by erm gene, which can lead to structural change of ribosome and make the *Streptococcus agalactiae* resistant to erythromycin[26, 27].

In this study, the erythromycin resistance rate of the 149 isolates was 62.42%, counting for 93 strains. In the analysis of erythromycin resistance and positive rate of MLS_B resistance genes in *Streptococcus agalactiae*, mreA was the main MLS_B resistance gene, with a total of 87 strains (93.55%), 6 strains (6.45%) of ermA gene and 79 strains (84.95%) of ermB gene. The above results showed that the expression of mreA gene could lead to cross-resistance between erythromycin and other macrolide antibiotics. According to D-test, 127 Group B streptococcus strains of the 149 clinical isolates were resistant to erythromycin or clindamycin, including 52 strains (40.94%) of constructive MLS_B resistance (erythromycin resistance and clindamycin resistance, cMLS_B), 29 strains (22.83%) of induced MLS_B resistance (erythromycin resistance and clindamycin induced resistance, iMLS_B), 12 strains (9.45%) of active efflux resistance (erythromycin resistance but clindamycin sensitivity, MS), and 34 strains of L-phenotype of MLS_B resistance (erythromycin sensitivity or intermediate but clindamycin resistance, L).

Our evidence showed that almost all strains of Group B streptococcus clinical isolates containing ermB and mreA genes were resistant to erythromycin, indicating that the expression of the two kinds of resistance gene led to high resistance of GBS to erythromycin. Considering the positive rate of mreA and ermB genes in cMLS_B, 98.08% and 100% in MS type, the positive rate of the two genes in 29 strains of

GBS with iMLSB resistance phenotype was 44.8%. It indicated that the changes in ribosome structure of 50s methylase dominated by *ermB* gene and the active drug efflux mechanism brought by *mreA* gene may be involved in clindamycin-induced resistance of *Streptococcus agalactiae*, especially in MS and cMLSB type. Detection of *mreA* and *ermB* gene by PCR can be used to determine the resistance of patients to GBS. Recently, Guerin, F. have reported that *erm* (47) encodes a methyltransferase that adds a single methyl group to 23S nucleotide A2058 of H.Kunzii[28]. Among the 149 strains of GBS isolated in this clinical sample, 3 strains carried *erm* (47) gene by qPCR validation, suggesting that *erm* (47) was also transferred to animals. However, the expression of *erm* (47) gene was not significantly associated with MLSB resistance. The evolution trend of *erm* (47) in GBS among human population is still worthy of attention.

In sum up, *Streptococcus agalactiae* colonized in perinatal pregnant women in Xinjiang region has a high resistance level to erythromycin. Considering erythromycin is one of the most representative antibiotics of lincomycin, it indicates that patients with Group B Streptococcus infection in this region may have serious cross-resistance to MLS_B antibiotics. Therefore, the hospital should try to avoid repeated drug use in patients with clinical medication, and should also strengthen the detection of invasive *Streptococcus agalactiae* MLS_B resistance genes, keep monitoring the factors causing drug resistance, and understand the development and dissemination of pathogens, which is conducive to the prevention and control of *Streptococcus agalactiae* drug resistance.

Declarations

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Tables

Primers	Sequence	length
ermA-F	5'-CGATATTCACGGTTTACCCA-3'	513bp
ermA-R	5'-CGTAATAGAAATCGGATCAGGA-3'	
ermB-F	5'-ACCGATACCGTTTACGAAATTG-3'	372bp
ermB-R	5'-TTGCTGAATCGAGACTTGAGTG-3'	
mreA-F	5'-ACACCTCGTCTAACCTTCGCT-3'	431bp
mreA-R	5'-GTTTCGTCCTCTTAGCATCTCCAT-3'	
mefA-F	5'-GTGTGCTAGTGGATCGTCATGA-3'	389bp
mefA-R	5'-ACCCAGCTTAGGAATACGTACAAT-3'	
mefE-F	5'-CGTAGCATTGGAACAGC-3'	513bp
mefE-R	5'-TCGAAGCCCCCTAATCTT-3'	
lin B-F	5'-CCTACCTATTGTTTGTGGAA-3'	944bp
lin B-R	5'-ATAACGTTACTCTCCTATTC-3'	
erm47-F	5'-atcgattaatTAAGGAGGTCATAATATGCAAATATATG-3'	81bp
erm47-R	5'-gacctccttaATTAATCGATTGGTTTAATGAAC-3'	

Table 1. Primers and products lengths of erythromycin/clindamycin-resistance genes used in conventional PCR

Antibiotic/method	Susceptible	Intermediate*	Resistant
Kirby-Bauer disk diffusion			
linezolid	149 (100 %)	-	-
penicillin	149 (100 %)	-	-
vancomycin	149 (100 %)	-	-
meropenem	149 (100 %)	-	-
ampicillin	149 (100 %)	-	-
ceftriaxone	149 (100 %)	-	-
levofloxacin	67 (44.97%)	19 (12.75%)	63 (42.28%)
erythromycin	51 (34.23%)	5 (3.36%)	93 (62.42%)
tetracycline	84 (56.38%)	26 (17.45%)	39 (26.17%)
clindamycin	44 (29.53%)	19 (12.75%)	86 (57.72%)
* The intermediate values were assimilated to resistant			

Table 2. Susceptibility profile of GBS isolates (n=127)

Phenotype**	Strains*	Rate
cMLSB (E-R, C-R)	52	40.94%
iMLSB (E-R, C-S)	29	22.83%
M (E-R, C-S)	12	9.45%
L (E-S, C-R; E-I, C-R)	34	26.77%
* Among 149 isolated GBS, 127 strains showed positive macrolide resistance phenotype and 93 strains showed positive erythromycin resistance phenotype.		
** E=Erythromycin, C=Clindamycin, S=sensitive, I=immediate, R=resistant		

Table 3. Macrolide-lincosamide-streptogramin (MLS_B)phenotypes of GBS isolates(n=127) by D tests

Gene	Strains(n=149)	Strains(n=127)
ermB	119(79.87%)	109(85.83%)
ermA	9(6.04%)	9(7.09%)
mreA	135(90.60%)	118(92.91%)
LinB	40(26.85%)	40(31.50%)
mefA/E	96(64.43%)	94(74.02%)
erm (47)	3(2.01%)	3(2.36%)

Table 4. Statistical analysis of MLS_B resistance genes of GBS isolates

Phenotype*	strains/ (%)	genes(strains)
cMLS _B (E-R, C-R)	52(40.94%)	ermA (5), mreA (1), linB (36), mefA/E (46), ermB+mreA (51)
iMLS _B (E-R, C-S)	29(22.83%)	ermB (3), ermA (1), mreA (10), mefA/E (6), ermB+mreA (13), erm47(2)
M (E-R, C-S)	12(9.45%)	ermB+mreA (12), erm47(1)
L (E-S, C-R; E-I, C-R)	34(26.77%)	ermB (1), ermA (3), mreA (2), linB (4), mefA/E (15), ermB+mreA (29)

*E Erythromycin C Clindamycin, S sensitive I immediate R resistant

Table.5 Relationship of phenotype to genotype in phenotypically macrolide-resistance GBS isolates(n=127)

Figures



Figure 1

D-test of erythromycin resistant GBS strains of clinical isolates. A. The negative group of D-test with no change of the inhibition zone of clindamycin which represents the active efflux resistance, MS type with erythromycin resistance but clindamycin sensitivity. B. The result of D-test with no inhibition zone of erythromycin and clindamycin represents the intrinsic drug-resistant of GBS, which indicates a resistance phenotype of constructive MLS_B (cMLS_B). C. The positive group of D-test shows a D inhibition shape which represents the iMLS_B phenotype with erythromycin resistance and clindamycin induced resistance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables of GBS multidrug resistance gene.xlsx](#)
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